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Abstract

MicroRNAs (miRNAs) are post-transcriptional inhibitory regulators of gene expression by binding to complementary messenger RNA (mRNA) transcripts. Extracellular miRNAs are transported by membrane-derived vesicles (exosomes and microparticles), lipoproteins, and other ribonucleoprotein complexes. Extracellular microRNA are emerging as important mediators of intercellular communications, being involved in the transmission of biological signals between cells. Several miRNAs have been identified as having a primary impact on many biological processes that are of direct relevance to cardiovascular complications of diabetes. Whether the extracellular miRNAs are directly involved in the regulation of these processes is yet to be established. Here, we review recent progresses in extracellular miRNA biology and the role of extracellular miRNA in diabetes induced cardiovascular disease, describing the regulators affecting miRNA transport and the mechanisms for different miRNA transporters. In addition, we discuss the advancement of the research in this field and identify the associated challenges.

Keywords: MicroRNAs; Extracellular vesicles; Exosomes; Diabetes; Cardiovascular disease.

Introduction

Diabetes mellitus is a chronic metabolic disorder that is characterized by defects in both insulin secretion and insulin action. The disease is strongly associated with both microvascular (retinopathy, nephropathy, neuropathy, and microangiopathy in different tissues) and macrovascular (coronary artery disease –CAD-, peripheral arterial disease –PAD- and cerebrovascular disease) complications [1]. Diabetes is an independent risk factor for cardiovascular disease (CVD). Several epidemiological and clinical studies have contributed to show higher prevalence of heart disease among diabetics as compared to non-diabetic subjects. Moreover, due to the presence of concomitant risk factors, diabetics tend to develop heart disease at an earlier age [2]. However the asymptomatic nature of the disease makes early detection difficult. The prevalence of diastolic dysfunction is as high as 40-60% in type 2 diabetics [3]. Moreover, the prevalence of myocardial dysfunction and heart failure (HF) was almost twice in diabetics [4]. Interestingly even after reducing other risk factors such as hypertension, CAD and obesity, diabetes still remained as an independent risk factor for development of CVD [5]. Prevalence of diabetes has been shown to enhance fatty acid metabolism, impairing glucose oxidation and intracellular signalling eventually leading to adverse cardiac remodelling [6].

There are sparse evidences, including by us, that intracellular and extracellular microRNAs (miRNAs) play important roles in regulating diabetes and diabetes mimicking conditions [7-10].

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 22-nucleotides, which have increasingly been recognized as potent post-transcriptional regulators of gene expression [11]. The specificity of miRNA targeting is generally defined by the complementarities between positions 2 to 8 of miRNA 5'-end (also termed the seed sequence) with the 3' untranslated region of target messenger RNAs (mRNAs). The capacity of miRNAs to simultaneously inhibit many different mRNAs allows for an amplification of the biological responses emanating from miRNA expressional changes [12].

The surprising observation that some miRNAs are also abundantly present in cell-free blood derivatives such as plasma and serum was made by several independent research groups (reviewed [13]). Being embedded in extracellular vesicles (EVs), such as exosomes, microvesicles and apoptotic bodies, partially justifies the resistance of miRNAs released

outside the cell from nucleases [14, 15]. The assumption that extracellular miRNAs are mainly present in body fluid encapsulated in exosomes and microvesicles was challenged by several studies, which found that a huge fraction of miRNAs exported by cells in culture are also associated with proteins and protein of the Argonaute (Ago) family [16, 17]. However, we believe that both mechanisms of miRNAs release are important and not necessarily mutually exclusive (for example EVs could embed miRNA-Ago complexes).

Cell-to-cell communication is necessary for proper coordination, both during development and among different cell types within adult tissues. Before the research field of EVs exploded in the last years, cells were already known to communicate *via* secreted molecules, by cell surface molecules, or by direct cell-to-cell contact allowing for exchanges of cellular contents *via* different kind of channels and pores [18]. The concept that miRNAs are transported into extracellular spaces, together with the evidence that exchange of miRNAs between cells can be accomplished through extracellular transfer, led to a revolutionary hypothesis of the existence of an intercellular communication system by extracellular miRNAs.

The present review focuses on the definition of extracellular miRNAs and examines evidence for their mechanism of transport outside and inside the cell. We will discuss recent studies that indicate extracellular miRNAs as novel mediators of intercellular communication, particularly as it relates to diabetes-induced cardiovascular complications.

1. miRNA and complications of diabetes

1.1 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is described as the diabetes-associated changes in the structure and function of the myocardium that is not directly linked to other confounding factors such as coronary artery disease (CAD) or hypertension[19]. We have previously reported upregulation of miR-1 and a consequential decrease in one of its target Pim-1 associated with the development of cardiomyopathy in STZ-induced type 1 diabetic mice [20]. Systemic administration of Pim-1 using an adeno-associated virus serotype-9, stopped the progression of cardiomyopathy through promotion of a pro-survival signalling [20]. Another study by Yildirim *et al* showed downregulation of miR-1 along with miR-499, -133a and -133b and upregulation of -21 in STZ-induced type 1 diabetic rats[21]. Intervention with an anti-oxidant (N-acetylcysteine) for 4 weeks helped to normalise the cardiac function along with restoration

of the above mentioned miRNAs suggesting an inverse relationship of these miRNAs with oxidative stress in diabetic heart[21]. The contrasting reports from this two independent studies could be due to the difference in the species and also due to the different time points where the levels of miR-1 was measured[22]. As the diabetic cardiomyopathy progresses an increase in myocardial insulin resistance is observed, resulting in downregulation of glucose transporter 4 protein (GLUT 4). Lu *et al* reported upregulated expression of miR-223 to be associated with GLUT 4 expression in insulin-resistant heart[23]. In diabetic human and mouse heart, the expression of GLUT4 is downregulated. However GLUT4 expression was increased by miR-223 [23]. The authors speculate this as an adaptive response to restore normal glucose uptake in the pathophysiological condition[23]. Another complication associated with diabetic cardiomyopathy is mitochondrial dysfunction leading to increased oxidative stress and decrease in ATP production[24]. It was reported that miR-141 upregulation modulated Slc25a3 gene which catalyses the transport of phosphate into mitochondrial matrix in cardiomyocytes of type 1 diabetic mice[24]. This could be a potential mechanism for low ATP production in diabetic heart. Collectively the studies show important implication of miRNAs in diabetic cardiomyopathy.

1.2 Diabetic Nephropathy

Diabetic nephropathy (DN) is one of the most important complications of diabetes mellitus, with 50% of diabetics requiring painful and costly dialysis [25]. DN is characterised by hypertrophy in the glomerular mesangium and tubular compartments along with podocyte dysfunction and ECM protein accumulation. Signalling pathways such as transforming growth factor-b (TGF-β) [26], Phosphoinositide 3-kinase-protein kinase B (PI3K-Akt) [27], Mitogen-activated protein kinase (MAPK) family including P38, extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinases pathways have been reported to result in glomerular hypertrophy and ECM accumulation in DN [28-32]. Recent studies have demonstrated the role of miRNA in regulating the signalling pathways in the pathogenesis of DN. For example, increased expression of miR-192, miR-200b/c, miR-216a and miR-217 in mesangial cells (MCs) in type 1 (streptozotocin injected) and type 2 (db/db mice, leptin receptor mutant) diabetic mouse models [33-36]. miR-192 targets E-box repressors, ZEB1/2 in MCs to activate TGF-β leading to renal fibrosis proteinuria [37]. Interestingly this also result in upregulation of other miRNA in MCs such as miR-216a/miR-217 and miR-200b/c thereby increasing the collagen

expression in DN [37]. miR-216a/217 is reported to activate Akt kinase by targeting Pten in MCs treated with TGF-β1 enhancing cellular hypertrophy [34]. miR-200b/c is also reported to activate Akt by targeting FOG2, an inhibitor of PI3K [36]. miR-21 was reported to target phosphatase and tensin homolog (PTEN) to induce the overactivation of Akt signalling pathway, followed by renal fibrosis and hypertrophy [38]. In db/db mice, miR-21 increased TGF-1 signalling by targeting Smad7; thus, an inhibitor of miR-21 was evaluated as a therapeutic target in this model [39]. These DN-inducing miRNA were found to be overexpressed in diabetic kidney and inhibiting these miRNA showed less severe phenotypes of DN in mice. For example, miR-21 knockdown by LNA-anti-miR-21 in diabetic kidneys of db/db mice significantly reduced microalbuminaria, renal fibrosis and inflammation [39]. In another study, LNA-anti-miR-192 attenuated DN in C57 type 1 diabetic mice by restoring the function of Zeb1/2 thereby downregulating ECM genes [40]. Also, miR-192-knock out mice were found to be protected from the key features of DN [41].

Studies have shown that several DN-inducing factors such as TGF-β2, COL1, COL4, and NADPH oxidase subunit 4 (NOX4) are also targets of number of miRNA which are downregulated in DN. For example, all three members of the miR-29 family (miR-29a,b,c) were found to be downregulated in proximal tubular cells (NRK-52E), primary mouse mesangial cells, and human podocytes under high glucose/ TGF-β1 conditions[42, 43]. MiR-29 family directly targeted 3'UTR of the fibrotic genes, COL1 and COL4 resulting in their downregulation[43]. miR-29b is reported to have a renal protective role in db/db mice by inhibiting TGF-β/SMAD3 signalling pathway and specificity protein 1/NF-κB-driven renal inflammation[44]. Fu *et al* reported significantly reduced levels of miR-25 in kidneys from diabetic rats and high glucose-treated mesangial cells along with increase in NOX4 expression[45]. NOX4 levels could be restored by inhibiting miR-25. Lucifarase assays showed miR-25 directly binding to 3'UTR of NOX4 mRNA[45]. Collectively these studies show several miRNA are involved in regulating multiple biological effects in DN, with some taking part in the pathogenesis and development of the disease while others as DN preventers. These upregulated or downregulated miRNA may be potential targets for the treatment of DN.

1.3 Diabetic Retinopathy

Diabetic retinopathy (DR) is a major cause for loss of vision as well as visual disabilities in adult population in developed countries. The disease is characterized by impairment of blood

vessels in retina, which, upon proliferation, leads to proliferative diabetic retinopathy (PDR); the abnormality of retinal blood vessels can also result in loss of function with leakage of fluid and lipids in retina as a consequence. In the case, when edema, caused by accumulation of fluid, affects central retina or macula, the end result could be blindness. Other consequences of DR include increased permeability of the retina, retinal ischemia and retinal neovascularization [46].

During diabetes, hyperglycemia leads to cellular damage on vascular and neuronal levels via a number of mechanisms: elevated oxidative stress, activation of the protein kinase C (PKC) pathway, increased inflammation, production of advanced glycation end-products (AGE) and osmotic stress caused by accumulation of sorbitol through polyol pathway [47-50]. Furthermore, Abu El-Asrar et al. has reported that retinal endothelial cells undergo endothelial-to-mesenchymal transition, which promotes pathologic fibrosis in PDR [51].

The first evidence of miRNA involvement in pathogenesis of DR comes from Kovacs et al. study, where it has been demonstrated that there is a significant change in miRNA expression profile in retinas and retinal endothelial cells (RECs) between diabetic and control rats [52]. Specifically, expression of 80 miRNAs was upregulated while 6 miRNAs were downregulated in retinas of diabetic rats; moreover, 16 miRNAs were significantly increased whereas 104 miRNAs were reduced in RECs of diabetic rats vs the control ones [52]. What is more, out of the 16 elevated miRNA in RECs 4 (miR-146, miR-155, miR-132, and miR-21) are induced by NF-κB, which is the main player in cellular inflammatory response and is also a significant pathological driver of early DR [52, 53]. Furthermore, it has been reported in another study that miR-200b expression is downregulated in the retinas of STZ-induced diabetic rats while its direct target gene, VEGF, was upregulated in the same condition, which leads to the leakage of blood vessels [54]. On the other hand, Murray et al. demonstrated that miR-200b levels are increased in retinas of genetically-induced diabetic (Akita) mice with the reduction of its target's, Oxr1, expression that exerts protection against oxidative stress [55, 56]. The opposing effects between the two studies might have arisen due to the fact there is a difference between STZ-induced and genetic model of diabetes. Interestingly, Feng et al. reported that hyperglycemia reduced the expression of miR-146a and, thereby, caused an overexpression of p300 transcription factor that resulted in accumulation of an extracellular matrix protein, fibronectin, in rats' retinas, which occurs during retinopathy [57]. Further, it has been shown that oxygen-induced retinopathy (OIR) in mice brings the levels of miR-126

down, thereby increasing the expression of VEGF, IGF-2 and HIF-1α via upregulation of p38 and ERK signalling proteins; this leads to retinal neovascularization, which can result in blindness during diabetic retinopathy [58]. Finally, Mortuza et al. demonstrated that there is an elevation of miR-195 levels in retinas of STZ-induced diabetic rats that reduces the expression of SIRT1 and, consequently, upregulates fibronectin content; moreover, miR-195-induced downregulation of SIRT1 promotes ageing-like changes in diabetic retinas of the rats [59].

1.4 Diabetic Neuropathy

Diabetic peripheral neuropathy (DPN) is nerve damage occurring in the presence of diabetes, with prevalence greater than 50% in patients with long-standing disease. The typical DPN is a chronic, symmetrical, length-dependent sensorimotor polyneuropathy [60]. Total hyperglycemic exposure is perhaps the most important risk factor and it is associated with metabolic derangements and cardiovascular risk factors [61]. Alterations of microvessels appear to be associated with the pathologic alterations of nerves [62]. Numerous studies try to elucidate the underlying mechanisms of this disease. Several reports have demonstrated that a variety of molecules are likely involved in the development of diabetic neuyropathy, such as protein kinase C, polyol, aldose reductase, advanced glycation end-products, reactive oxygen species, cytokines [63]. Only recently DPN has been associated with miRNA regulation. miR-146a has been demonstrated to protect dorsal root ganglion neuros (DRG) from hyperglycaemia-induced apoptosis thought regulation of TRAF6 and IRAK1 pathway. Interestingly, the administration of sildenafil, a phosphodiesterase type 5 inhibitor, in a mouse model of diabetic neuropathy increased miR-146a expression and the survival of DRG neurons [64]. On the other hand, Increased expression of miR-146a and NF-κB, and a twofold decrease in the expression of TRAF6 were observed in the sciatic nerve of diabetic rats [65], showing that same miRNA could have opposite function in neurons from different tissues. Pro-survival activity of DRG in a rat model of diabetes has been also associated with upregulation of miR-29b and inhibition of SMAD3 [66].

Two studies using miRNA sequencing were able to identify miRNAs that were differentially regulated in neuropathy. DRG miRNA microarray identified significant changes in expression let-7i and miR-341 in diabetic mice [67] . Analysis of microRNA expression in the lumbar spinal dorsal horn of STZ-induced diabetic neuropathic pain (DNP) mice showed that miR-184-5p

and miR-190a-5p are highly expressed in DPN tissues and they act as regulator of inflammation [68].

Polymorphisms have been identified in miR-128a, miR-27a and miR-146a and there were associated with susceptibility for diabetic polyneuropathy and for cardiovascular autonomic neuropathy [69].

1.5 Pancreatic β-cells

Pancreatic β-cells play a fundamental role in glucose homeostasis, releasing insulin in response to glucose levels in the bloodstream. Absence or malfunction of β-cells leads to type-1 diabetes (T1D), due to lack of insulin producing cells or type-2 diabetes (T2D), characterized by the inability to increase insulin levels to sufficiently stimulate glucose uptake [70]. Expression profiling experiments identified a large set of miRNAs has been implicated in β-cell development, including miRNA belong to the miR-16 family (miR-15a/b, miR-16, miR-195)[71], miR-503, miR-541, miR-214 [72], miR-9, miR-7, miR-376 and miR-375 [73], among them.

miR-375 has been identified as specific β-cells miRNA in T2D patients and in the pancreatic islets of obese diabetic mouse models (*ob/ob*)[74]. Expression of miR-375 in adult β-cell islets is downregulated when high levels of glucose are available [75]. Low levels of miR-375 induce insulin secretion by de-repression of its targets Mtpn [76] and PDK1 [77] while overexpression of miR-375 exerts opposite effects on insulin secretion [77].

Stoffel group, using genetic approaches, showed that conditional deletion of miR-7a resulted in improved glucose tolerance by increasing β-cell secretion of insulin [78]. Interestingly, modulation of miR-7a expression in β-cells did not affect proliferation and apoptosis, showing that miR-7 is dispensable for the maintenance of endocrine β-cell mass. The same Authors has demonstrated that miR-200 family is strongly induced in islets of diabetic mice [78]. Furthermore, β-cell–specific overexpression of miR-200 in mice is sufficient to induce beta cell apoptosis. Conversely, mir-200 ablation in mice reduces β-cell apoptosis and ameliorates T2D. Regulation of an anti-apoptotic and stress-resistance network that includes the protein Dnajc3 and the caspase inhibitor Xiap is at the base of miR-200 family mechanism [78].

2. Transporters for extracellular miRNAs

Extracellular miRNAs can be found in high concentrations in body fluids, where they maintain good stability despite the high RNase activity indicating that some mechanisms are in place to confer the miRNAs protection from digestion [79-81]. Indeed, naked miRNAs added to plasma are degraded in <5 sec, whereas circulating miRNAs are stable for many hours under the same conditions [82]. This phenomenon reflects the presence of an endogenous mechanism for the remarkable stability of miRNAs. The miRNA resilience may be explained by them being packaged in membrane encapsulated vesicles including exosomes[83], microvesicles and apoptotic bodies[84] and/or be protected by RNA-binding proteins [85] that offer protection against RNase activity.

2.1 miRNAs transport via exosomes

Exosomes are a subtype of membrane vesicles of approximately 30-100nm in size that are released from the endocytic compartment of live cells [86]. Endosomes originate from inward budding of the cytoplasm membrane. Surface proteins of plasma membrane may be transferred to the inner membrane of the endosomes during this process. Invagination of endosomes generates intraluminal vesicles (ILVs) or exosomes with cytoplasmic components or transmembrane proteins within them. Endosomal sorting complex required for transport (ESCRT), mediates budding of ILVs[87]. Accumulation of exosomes in the lumen of endosomes results in multivesicular bodies (MVBs). MVBs can either fuse with lysosomes to follow a degradation pathway or fuse with plasma membrane releasing exosomes to the extracellular space [88]. Accumulation of specific molecules such as ceramide and Ca2+ dependent scramblase favours pinching off the membrane and eventual release of the contents into the extracellular space (reviewed in [89]). Although initially considered as cell debris, exosomes have now emerged as important regulators of biological functions via transfer of genetic materials. Once released, exosomes can either target neighbouring cells or enter the blood stream, thus potentially reaching distant cellular targets. In 2002, exosomes were first reported to transfer information between cells but it was not until 2007 that a seminal study by Valadi *et al* proved exosomes to contain functional miRNAs which can be delivered to another cell [83]. MiRNA-214 (miR-214) plays a dominant role in exosome-mediated signalling between ECs. EC–derived exosomes stimulated migration and

angiogenesis in recipient cells, whereas exosomes from miR-214–depleted ECs failed to stimulate these processes [90].

In a recent study, authors performed a deep sequencing analysis on small RNAs isolated from ECs and their corresponding exosomes [91]. The authors demonstrated that different RNA classes show different distributions between ECs and exosomes. An in-depth analysis of miRNA sequences shows that 5p, 3p and stem-loop fragments of identified miRNAs are differentially distributed between cells and exosomes. In addition, exosomes are enriched with miRNA-like fragments derived from snoRNAs, vault RNA, yRNAs and the degradation products of long coding RNAs [91]. These findings suggest that, besides possible targeted transport of small RNAs to exosomes, there is a mechanism for localized degradation of RNA in the proximity of multivesicular bodies and the subsequent disposal of RNA fragments through exosomes [91].

2.2 miRNAs transport via apoptotic bodies

Cell apoptosis is an important mechanism by which unnecessary cells are removed in multicellular organisms. Cells undergoing apoptosis release small sealed membraneous vesicles termed as apoptotic bodies. Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without nuclear fragment and are subsequently phagocytozed by macrophages and degraded within phagolysosomes. [84, 92, 93].

Zernecke *et al* found that EC at atherosclerotic sites secreted apoptotic bodies into the circulation which where engulfed by phagocytes triggering secretion of cytokines or growth factors[84]. The apoptotic bodies secreted by the ECs showed unique signature of miRNAs[84]. One of the highest expressed miRNAs was miR-216, implicated as a key regulator of VEGF and FGF signalling in ECs[84]. The study showed that dying ECs communicate with neighbouring cells via apoptotic bodies packaged with specific miRNAs resulting in a healing vascular protective responses [84].

2.3 miRNAs transport via microvesicles

Microvesicles are all cell-derived vesicles that are enclosed by a lipid bilayer, ranging from 50nm to 1,000 nm in diameter depending on their origin. In contrast to exosomes that are derived from the endolysosomal pathway, microvesicles are generated by budding from the plasma membrane. Therefore, although they are heavily enriched in phosphatidylserine, the

membrane composition of microvesicles reflects that of the parent cell more closely than does the membrane composition of exosomes. Microvesicles are characterized by specific cytoplasmic proteins, certain lipid raft-interacting proteins and RNAs [94].

Zhang et al. demonstrated that miR-150 from monocytic cells was delivered into ECs via microvesicles, and reduced the expression of c-Myb, resulting in the increase of cell migration in EC both in vitro and in vivo [95].

In addition, another study also showed that the microvesicles from CD34+ peripheral blood mononuclear cells exhibited proangiogenic activity in ECs via the transfer of miR-126 [96]. In similar context, Cantaluppi et al. reported that EVs released from endothelial progenitor cells (EPC) contains miR-126 and miR-196 and enhanced islet EC proliferation, migration, anti-apoptosis, and angiogenesis [97].

A recent study demonstrated that platelets can remotely modulate vascular EC apoptosis through releasing miRNA-223-containing microvesicles. Exogenous platelet miR-223 can decrease the level of insulin-like growth factor 1 receptor and thus promotes HUVEC apoptosis induced by advanced glycation end products. Moreover, Ismail *et al.* found that EVs from macrophage contained miR-223, and transport miR-223 in a functionally active status to target cells, including ECs [98].

2.4 miRNAs transport via proteins and lipoproteins

Nucleophosmin 1 (NPM1) and Argonaute 2 (Ago2) are two proteins that are confirmed as miRNA carriers. NPM1 is a ubiquitously expressed nucleolar protein involved in exporting RNAs and ribosomal proteins to cytoplasm[99]. The Ago2-miRNA complexes forms a main part of RISC complex during gene regulation and they are present in human plasma[100]. However it still needs to be confirmed if this is the intracellular Ago2-miRNA complex or whether this originates from disassembled Ago2 loaded with a new mature miRNA when already in the extracellular compartment. Extracellular miRNAs are also transported by high-density lipoproteins (HDL) and low-density lipoproteins (LDL). It was initially showed that lipoprotein-bounded miRNAs were not very different in CAD patients and they could not be easily transferred to vascular cells and circulating monocytes[101]. However, later studies by Vickers *et al* rather suggested that the HDL-miRNA profile of normal subjects is significantly different from that of people with familial hypercholesterolemia [85]. This is in line with the concept that HDL can attracts different molecular partners in health and disease, thus eliciting

variable effects on vascular function dependently on those[102] [103]. In particular, HDL from type 2 diabetes mellitus or metabolic syndrome patients cannot reproduce the endothelial-protective effects similar to the HDL from healthy subjects[104]. Additionally, reconstituted HDL attracted different miRNAs when injected into the systemic circulation of atherosclerotic mice in comparison to healthy mice. The latter suggest that HDL can bind with miRNAs after the miRNAs have been released in the circulation by the producing cells [85]. The functional importance of HDL-conjugated miRNAs is suggested by studies where HDL-miRNAs from atherosclerotic or control subjects were used to stimulate hepatocytes cells, which responded with differential gene expression changes [85]. The same study also showed that the cellular export of miRNAs to HDL is regulated by neutral sphingomyelinase and that the HDL delivery of miRNAs to recipient cells is mediated *via* a scavenger receptor class B type I [85]. HDL delivery of both exogenous and endogenous miRNAs resulted in the direct targeting of mRNA reporters. [85]. Taken together, the observations of Vickers *et al* indicate that HDL transport and deliver miRNAs, with functional consequences on the recipient cells and that the miRNAs transported by HDL could be responsible for the heterogeneity in vascular response to HDL. It would be of extreme importance to expand the HDL-miRNAs studies to the diabetes setting with a precise cardiovascular focus.

3. Mechanisms of miRNAs export and transfer

3.1 Selective miRNAs export in extracellular vesicles

It is emerging that the quality and concentration of miRNAs contained in EVs is dictated by the type of producing cells as well as by forced changes in their molecular profile and exposure to different environmental conditions. For example, EVs secreted by KLF2-transduced or shear-stress-stimulated endothelial cells are enriched in miR-143/145 [105]. The vesicular export mechanism for miR-143 induced by the shear stress responsive transcription factor KLF2 in endothelial cells has been recently suggested to depend on Rab7a/Rab27b, two GTPases involved in the coordinators of membrane trafficking[106]. For what concerns the setting of diabetes cardiovascular and renal complications, we showed that miR-503 is contained in microparticle secreted by ECs, but not by pericytes and that diabetes-mimicking culture conditions increase the abundance of miR-503 in the EC-released microparticles [8].

Moreover, cardiac myocytes from type 2 diabetic rats release exosomes with higher miR-320 in comparisons to cells from non-diabetic controls [107]. These studies showed that both the aforementioned alterations provoke detrimental functional consequences on the recipient vascular cells, namely pericytes and EC, respectively [8] [107]. Exosomes from kidney cells are also affected by exposure to high D-glucose (HG) concentration. For example, miR-145 increased in both cultured mesangial cells and their exosomes in response to exposure to HG [108].

The mechanisms whereby miRNAs are sorted to EVs are not known, if not very preliminary. Some evidence of a non-random sorting of miRNAs in exosomes comes from Guduric-Fuchs *et al.*, who found that a specific set of miRNAs, such as miR-142-3p, miR-150 and miR-451, are preferentially sorted in exosomes from HeLA cells [109]. Moreover, Squadrito *et al* performed RNA profiling of macrophages and their exosomes and presented evidences that miRNAs sorting to exosomes is modulated by changes of the miRNA target mRNAs in the cells. They went on to show that perturbing the expression of individual miRNAs or their targeted mRNAs promotes bidirectional miRNA relocation from the cell cytoplasm/P bodies to MVBs, thus controlling miRNAs sorting to exosomes [110]. Equivalent studies in cultured cardiovascular cell types exposed or not to diabetes or diabetes mimicking conditions are still lacking. In vivo studies showed that the exosomal miRNAs in type 1 diabetic patients is affected if the patients have incipient diabetic nephropathy. In details, miR-130a and miR-145 were enriched, while miR-155 and miR-424 were reduced in urinary exosomes from diabetic patients with microalbuminuria [108]. The miR-145 data were confirmed using an animal model of diabetic nephropathy, where miR-145 was also found increased in the glomeruli [108]. Interestingly, the pool of exosomal miRNAs circulating in diabetic subjects might be regulated by life style improvements, such as regular exercise [111]. In fact, Chaturvedi *et al* found that exosomal miR-455 and miR-29b increase during exercise in *db/db*, a mouse model of type 2 diabetes. Both miRNAs target metalloprotease-9 (MMP9), which has damaging effects on extracellular matrix remodelling. In line, tissutal MMP9 was found reduced in active mice [111]. Together these above reported studies suggest the existence of regulatory mechanism(s) determining the miRNA exosomal sorting, which can be altered by different physio-pathological conditions.

3.2 Mechanism of miRNAs loading into exosomes

To this date only a few main mechanisms of miRNA loading into exosomes are described in a certain degree of detail. The first system is based on neural sphingomyelinase 2 (nSMase2)-dependent pathway. The nSMase2 enzyme that regulates ceramide biosynthesis. The earliest evidence comes from Kosaka *et al*, who found that nSMase2 inhibition resulted into decreased secretion of miRNAs *via* exosome, while miRNA secretion was enhanced by elevating the activity of nSMase2 [112]. It has been then was confirmed that overexpression of nSMase2 increased levels of miRNAs in exosome and the downregulation of the enzyme reduced the number of exosomal miRNAs [113]. Another mechanism of miRNA sorting into exosomes is dependent on miRNA motif and sumoylated heterogeneous nuclear ribonucleoproteins (hnRNPs). In detail, it was found that specific short miRNA sequences, called the EXOmotifs, determine the miRNA localisation into exosomes [114]. Moreover, it was identified that a particular member of hnRNPs, hnRNPA2B1, which has been previously demonstrated to coordinate mRNA trafficking to axons in neural cells [115], binds to the EXOmotifs on miRNA, thus driving the miRNA loading into exosomes [116]. The hnRNPA2B1-dependent miRNA sorting in exosomes is mediated by sumoylation of the protein [116].

In another study Koppers-Lalic et al. demonstrated that there is 30% difference in the amount of miRNAs in the total small RNA content between the cellular (50%) and exosomal (20%) fraction of B cells with a discordant miRNA distribution between exosomes and the intra-cellular compartment [117]. Specifically, it was found that miRNAs that are adenylated in their 3’ end are over-represented in the cellular fraction, whereas 3’ end uridylated miRNAs are enriched in the exosomes [117]. This study suggests that miRNAs with a sequence that contains uridines at 3’ end are preferentially loaded in exosomes, which is another evidence of the exosomal orting signal [117]. The aforementioned mechanisms are represented in **Figure 1.**

Another mechanism of exosomal miRNA loading involves the miRNA-induced silencing complex (miRISC) pathway. In particular, it has been reported that exosomes contain Ago2, which is a part of miRISC and mediates interaction between mRNA and miRNA [118]. Moreover, in HEK293T cells, the Ago2 knockdown led to reduction in the exosomal export of a miRNA pool, such as miR-451, miR-150, and miR-142-3p [109]. Further, it has been

demonstrated that Ago2 and another miRISC component of miRISC (GW182) associate with MVBs [119]. Lee *at al*. reported that inhibition of MVB formation results in decreased numbers of miRISC associated with P-bodies, which are physically attached to MVBs [120]. In addition, Squadrito *et al.* demonstrated that activation of bone marrow-derived macrophages via treatment with IL-4 led to differential distribution of miRNAs in exosomes [110]. In detail, the study shows that the reduction in cellular levels of the target mRNAs of specific miRNAs in response to cellular activation leads to increase in the number of those miRNAs in exosomes, thus suggesting that miRNA sorting in exosomes is a feedback mechanism, which balances the cellular levels of miRNA and its targets [110].

Cha *et al* proposed another mechanism of miRNA loading into exosomes based on KRAS protein, a GTPase which is encoded by an oncogene that is often mutated in colorectal cancer cells [121]. It has been found that the presence of mutant *KRAS* allele in the cell affects sorting of a particular set of miRNAs in exosomes. The study demonstrates that miR-100, which negatively regulates metastatic cancers, is downregulated in mutant *KRAS* cells, but elevated in the exosomes derived from the same *KRAS* cells, implying that cellular miR-100 decrease is a result of its sorting and secretion in exosomes [121]. It was reported that the inhibition of the nSMase resulted in three-fold increase in intracellular miR-100 levels, suggesting that the enzyme plays an important part in KRAS-dependent miRNA exosomal sorting [121].

In the *in vivo* cardiovascular setting, it was shown that CAD is associated with decreased miRNAs loading into MPs [122]. Data in the context of diabetes are still lacking and more research is needed.

3.3 Cell-to cell communication of extracellular miRNA

MiRNAs, which are secreted via EVs, are thought to exhibit cell-to-cell communication *via* paracrine and endocrine routes. There are two main known functions of the secreted miRNA in EVs: the first one is a conventional downregulation of the target genes in the recipient cells; the second function involves activation of the immune response by EV interacting with immune cells. There are several examples of the first mechanism. Zernecke *et al* found that apoptotic ECs in atherosclerotic plaques secrete miR-126 within the apoptotic bodies to target surviving recipient ECs, thus exerting protection from further atherosclerotic damage *via* downregulation of the miR-126 target RGS16 expression and recruitment of Sca-1+ progenitor cells [123]. Another study has illustrated that miR-143 and miR-145 secreted by

EVs from ECs under shear stress are taken up by the smooth muscle cells (SMCs), where they bring about the downregulation of their target genes, inducing atheroprotective effects [105]. Recently, the Baker group has identified the presence of miR-143-3p in pulmonary artery SMCs (PASMCs)-derived exosomes. Using assays with pulmonary arterial ECs (PAECs), they also demonstrated a paracrine pro-migratory and pro-angiogenic effect of the miR-143-3p enriched exosomes from PASMCs [124]. Moreover, Finn *et al* demonstrated that serum MPs from CAD patients show a deficiency in a glycoprotein (developmental endothelial locus-1: Del-1), which mediates the uptake of MPs by ECs. This caused a reduced uptake of MP-embedded miRNAs (miR-17, miR-19a, miR-21, miR-92a, miR-146a, miR-222, and miR-223) in recipient cultured ECs [122]. We have recently shown that in mice with diabetes and limb ischemia, the secretion of miR-503 in endothelial MPs is increased *via* a $p75^{NTR}$-dependent mechanism. $P75^{NTR}$ is an atypical neurotrophin receptor, which we already showed to be induced by diabetes in ECs with detrimental functional consequences *in vitro* and *in vivo* [8, 125, 126]. In cultured ECs, $p75^{NTR}$ activates the NF-kB signalling, which in turn induce miR-503 transcription and the MP shedding by triggering the expression of Rho kinase [8]. Intriguingly, MPs containing miR-503 are taken up by co-cultured pericytes, which respond with the downregulation of the miR-503 target genes, EFNB2 and VEGFA, and finally an impairment in their migration and proliferation capacities [8]. This study suggests how the crosstalk between different types of vascular cells under diabetes is modulated by miRNAs secreted in EVs. As an example of the immune system-mediated EV actions come from Fabbri *et al*, who reported that exosomal miR-21 and miR-29a from lung cancer interact with and activate toll like receptors (TLRs) on surrounding macrophages, which leads the immune cells to secrete prometastatic cytokines resulting in the growth and spread of the tumour. This is a novel example of tumour-immune cells communication [127].

More recent literature suggests an involvement of cancer-derived exosomes in determining the site of metastatic growth [128]. *Hoshino et al* demonstrated that exosomes from tumor cells with a differential tropisms (lung, liver, or brain) fuse preferentially with resident cells at their predicted destinations to prepare the pre-metastatic niche. Exosome proteomics revealed distinct integrin expression patterns, in which the exosomal integrins α6β4 and α6β1 were associated with lung metastasis, while exosomal integrin αvβ5 was linked to liver metastasis [128]. Integrins targeting in the exosome producing cells could decrease metastatic growth. In additional, clinical data indicate that exosomal integrins could be used

to predict organ-specific metastasis in patients. The role of exosomal miRNAs has not been investigated in this study, but miRNAs could contribute to the metastatic trigger once delivered *via* exosomes. These new concepts could be relevant also in the context of diabetes and cardiovascular disease, for example for predicting the organs that will be targeted by complications in patients with diabetes mellitus. Of course, more research is needed to evolve from these hypotheses.

4. Role of extracellular miRNA in diabetes-induced cardiovascular disease

MiRNAs transport via EVs has been implicated with the development of diabetic cardiovascular complications. For example, while cardiomyocytes from healthy rats were shown to promote angiogenesis responses *in vitro*; the same cell type cultured from type 2 diabetic rats inhibited the proliferation and migration of co-cultured ECs *in vitro*. These detrimental effects were eliminated when treated with exosome inhibitor GW4869 [129]. Further investigations showed an increased expression of miR-320 in exosomes extracted from diabetic rat cardiomyocytes[129]. Importantly, the exosomal miR-320 can be transferred to the EC leading to inhibition of angiogenesis favouring responses[129].

Recent evidences suggest that reduced miR-126 expression levels are partially responsible for impaired vascular repair capacities in diabetes (Zampetaki, Kiechl et al. 2010). Patients with type 2 diabetes showed a significant reduction in the level of vesicular miR-126, with non-vesicle-associated miR-126 was unchanged. Decreased vesicle enrichment of miR-126 was observed prior to the manifestation of diabetes and was correlated with severity of the disease. *In vitro* experiments in this study showed that exposure to HG reduced the miR-126 concentration in EC-derived apoptotic bodies. These data are in line with the publication from an independent laboratory. Jansen *et al.* also demonstrated that endothelial microparticles prepared from healthy subjects promote re-endothelialisation after endothelial injury in mice and EC migration and proliferation *in vitro* by transferring functional miR-126 to target ECs [130]. They also reported decreased miR-126 inside endothelial microparticles prepared from patients with stable CAD and diabetes. A recent study reported that first-trimester primary trophoblast cells when incubated with D-glucose (25mM) in hypoxic conditions significantly increased the release of exosomes[131]. These exosomes significantly increased the release

of cytokines such as granulocyte macrophage colony stimulating factor, IL-4, IL-6, IL-8, interferon-v and TNF-alpha from human umbilical vein endothelial cells (HUVECs) compared to controls[131].

We have previously identified that miR-503 expression is increased in ischemic limb muscles of type 1 diabetic mice and in ECs enriched from these muscles. Moreover, adenoviral administration of decoymiR-503 to the ischemic adductor of diabetic mice corrected diabetes–induced impairment of post-ischemic angiogenesis and blood flow recovery [7]. ECs and pericytes establish a cellular crosstalk in the microvasculature, which is fundamental for the regulation of angiogenesis, microvascular stabilization and permeability, but this protective mechanism is disrupted under diabetes. Our study established that under diabetic conditions, endothelial MPs carrying miR-503 interfere with ephrin-B2 and VEGFA expression in pericytes, further blocking post-ischaemic angiogenesis and vascular integrity [8].

Additionally, we have recently provided the first evidence that several miRs are differentially expressed in circulating pro-angiogenic cells (PACs) enriched from critical limb ischemia (CLI) patients with or without diabetes [132]. CLI-PACs showed higher levels of miR-15a and miR-16 and of the primary transcript pri-miR-15a/16a1. Overexpression of miR-15a and miR-16 impaired migration and survival in healthy PACs whereas inhibition improved the situation [132]. In addition we also provided preliminary evidence that in type 2 diabetic patients undergoing revascularization for CLI, increased circulating levels of miR-15a and -16 are associated with higher risk of limb amputation at one year follow-up, thus opening some scope for the use of extracellular miRNAs as predictive biomarkers in diabetic patients afflicted by severe cardiovascular complications [132].

Another group has reported that regenerative properties of circulating PACs were due to the transfer of exosomes enriched in miR-126 [133]. However exosomes from PACs isolated from blood samples of diabetic patients showed reduced miR-126 levels and impaired vascular repair potential [133].

We believe these aforementioned studies are of paramount importance to stimulate more fundamental research utilitarian to understand and then therapeutically manipulate the mechanisms leading to miscommunication between cells in the diabetes setting. Additionally, the possibility to develop extracellular miRNAs as predictive biomarkers of cardiovascular events in diabetic patients could help the monitoring and management of these patients,

which is currently far from optimal. Biomarkers studies will require work on large populations of diabetic patients that are well characterized at baseline and at different follow-up times.

5. The future of miRNAs transport

The findings described in this review detail the analysis of extracellular miRNAs along with providing a better understanding of miRNAs sorting to distinct secretory pathways and carriers. The regulation of miRNAs secretion and mechanisms of targeting is believed to generate opportunities to identify novel strategies for screening, monitoring and curing cardiovascular complications in diabetic patients.

Major achievements will be to determine which specific RNA sequences (if any) are necessary to export miRNA into the circulation and how diabetes could impact on these mechanisms. Comparative analysis of the sequences of miRNAs enriched in either cells or different types of EVs may provide information about possible mechanisms for selective incorporation of miRNAs into exosomes and other EVs and how these are affected under pathologies. Interestingly, miRNAs carrying an extensive 3' uracil residues added to their mature sequence have been proposed as important for selective incorporation into exosomes, whereas additional adenosines were detected at the 3' end of cellular retained miRNAs [117]. However, we cannot know if these potential regulatory mechanisms are maintained across cell type and under diseases and how these could explain changes induced by the environment or the cell type identity in the EV miRNAs cargo.

The export of miRNAs is likely to be regulated by cellular signalling. However, only few studies, described in the above section 2, have tried to start and investigate the regulatory mechanisms that govern the packaging of miRNAs into various carriers and miRNA secretion from cells. Therefore, the clarification of molecular mechanisms that regulate the secretion of miRNAs is a critical step for understanding their subsequent transport in the circulation. On the other side, we must admit that the investigatory tool-kits currently available for extracellular miRNAs and EV research are not optimized to address these ambitious questions and the development of new experimental approaches should parallel the investigation of our research questions in the regulatory mechanisms of extracellular microRNAs sorting and uptaking by recipient cells.

Of vital importance will be to discover the mechanisms that regulate extracellular miRNA stability. For example, protein typing of miRNA carriers, such as microvesicles and exosomes,

should facilitate not only the recognition of circulating EVs and their cellular origin understanding, but also the identification of protein factors contributing to the stability of miRNA in the circulation.

One of the key questions that need to be addressed is how extracellular miRNAs, whether encapsulated in EVs or in complexes with proteins, gain access to target tissues or cells. It is important to determine which receptor-mediated mechanisms exist between miRNA carriers and target cells to regulate the transfer of vesicular miRNAs. Recent evidences strongly suggest that integrins are involved in exosomes homing to different types of cells and organs [8, 128] Thus far the proposed mechanisms for how miRNAs are delivered to recipient cells depend on whether they are part of EVs (and what type of EV), lipoproteins, or Ago2 complexes. Receptor-mediated uptake would be expected for protein-associated miRNAs [134] and exosomes [135]. It is possible that part of the circulating Ago2-miRNA complexes are contained in EVs and use the vesicles as a shuttle system [119]. However, it is not clear how the recipient cells would take up Ago2-miRNA complexes, when these are not embedded in EVs or attached to lipoproteins. Importantly, receptors to transfer double-stranded RNA have been identified in mammals [136].

Because secreted miRNAs can be detected in biological fluids and can reflect the physiological status of the cells and organs they originate from, miRNAs could potentially serve as clinical biomarkers [137]. In order to reinforce the role of circulating miRNAs as biomarker of cardiovascular disease insurgence and evolution in diabetic patients, future studies will need to focus on deciphering the cellular origin of the extracellular miRNAs.

The challenges of miRNA targeting *in vivo* require the development of effective inhibitors that will decrease off-target effects. Naked miRNAs or anti-miRNAs are highly charged and often unstable in the circulation, thus packaging them into EVs can increase their potential for effective delivery and exciting therapeutic uses. EVs are better tolerated by the immune system than chemical nanoparticles as they represent natural transporters derived from endogenous cells. Moreover, this alternative approach could significantly reduce the dose of “miRNA drugs” and miRNA inhibitors necessary for treatment. Therefore, the investigation of the ability of such EVs carrying miRNAs to repair damaged tissues or vessels would be crucial for a future use in cardiovascular regeneration. For these studies, imaging techniques, innovative tracking systems and genetic animal models are required in order to assess the effectiveness of delivery of EVs in the target tissues.

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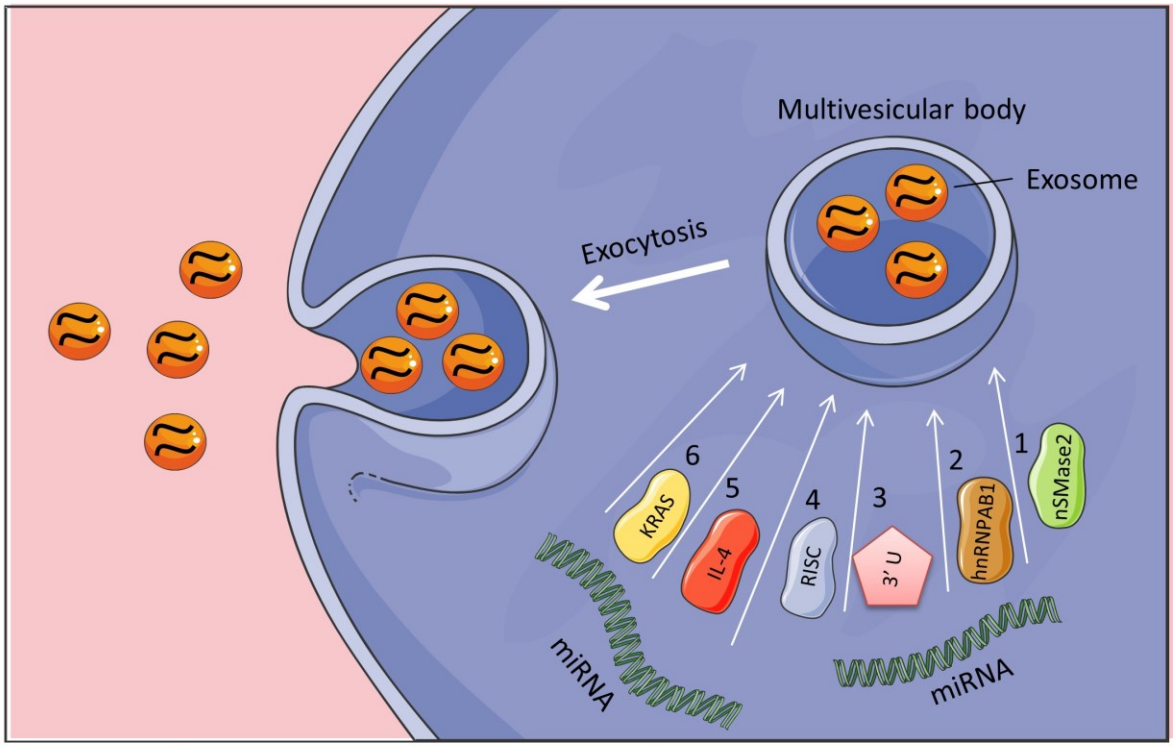
Figure



Number	miRNA sorting mechanism	References
1	nSMase2-dependent pathway	Kosaka et al., 2010
2	hnRNPA2B1-dependent pathway	Villarroya-Beltri et al., 2013
3	miRNA 3' end base modifactions	Koppers-Lalic et al., 2014
4	miRISC-dependent pathway	Melo et al., 2014; Guduric-Fuchs et al., 2012; Gibbings et al., 2009; Lee et al., 2009
5	IL-4 cellular activation-dependent pathway	Squadrito et al., 2014
6	KRAS-dependent pathway	Cha et al., 2015

Figure 1 | Mechanisms of miRNA sorting in exosomes. Cellular mechanisms, which regulate sorting of miRNA into exosomes